heart valves. Some reports have shown that patients with Hunter syndrome require regular follow-up assessment, such as electrocardiography and cardiac echocardiography, every 1–3 years [7, 9].

Before the development of ERT, some surgical cases of mucopolysaccharidosis were reported for valve replacement surgery. Bhattacharya et al. [2] and Antoniou et al. [1] reported mitral valve replacement surgery for mitral stenosis secondary to Hunter syndrome. Both patients in these previous reports were adults who had never received ERT.

Cardiac pathology of mucopolysaccharidosis has been studied more in type 1 than in type 2. Mucopolysaccharidosis type 1 cardiac valve pathology characteristically shows increased GAG content as well as infiltration of "clear" cells (Hurler cells) [3]. The reported patient had the pathologic finding of mucopolysaccharidosis type 2, similar to type 1, in the aortic valve. It has been suggested that Hurler syndrome and Hunter syndrome have a similar pathophysiology of cardiac valve disease.

Fesslova et al. [4] showed progression of cardiac valve disease in a patient with mucopolysaccharidosis types 1 and 2. In their study of eight patients who had cardiac valve disease and were receiving ERT, four patients had progressive disease, and the remaining four patients had stable disease.

For the reported patient, ERT was initiated after AR had already developed. Regardless of the ERT, cardiac valve disease deteriorated, and finally, AVR was performed. The finding of GAG accumulation in the aortic valve of the reported patient suggests that ERT does not reverse cardiac valve disease in Hunter syndrome. Therefore, ERT might not be effective after the onset of cardiac valve disease. Valve replacement surgery for a patient with Hunter syndrome during ERT has not been previously reported.

The actual mechanism of cardiac disease in mucopolysaccharidosis remains unknown. The heparin-, dermatan-, chondroitin-, and keratin-sulfate GAGs are normal components of cardiac valves. Accumulation of GAG is the main pathology of cardiac involvement in mucopolysaccharidosis, resulting in hemodynamic decongements [3].

We report, for the first time, AVR surgery for a patient with Hunter syndrome during ERT. In the reported patient, although mild cardiac valve disease had developed at the initiation of ERT, it was refractory to a 3-year course of ERT. This suggests that ERT has a limited efficacy for cardiac valve disease both clinically and pathologically. Antibody formation could hinder the effects of ERT. We hypothesize that idursulfase does not reach the cardiac valve because of its poor vascularity. Further study is required to determine the accurate pathophysiology of cardiac valve disease in patients with Hunter syndrome.

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Brief Communication

Enzyme replacement therapy in two Japanese siblings with Fabry disease, and its effectiveness on angiokeratoma and neuropathic pain

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ABSTRACT

Enzyme replacement therapy (ERT) for Fabry disease does not show a clear benefit in angiokeratoma. We describe two Japanese siblings with Fabry disease, who were diagnosed when angiokeratomas were found on the older sibling at the age of 13 years. Neither of the boys complained of pain, while both suffered from hypohidrosis. We evaluated the safety and efficacy of ERT with recombinant human agalsidase alfa (Replagal®, Dainippon-Sumitomo Pharma. Co., Osaka, Japan) in these siblings over a 5-year period.

In both siblings, sweating was observed 3 months after the initiation of ERT, which motivated them to adhere to ERT. Pain sensation was regained after 12 to 36 months of ERT, followed by a decrease after 48 to 60 months. Angiokeratomas on the lateral side of the knee of the older sibling partially disappeared after 48 months of ERT. Although the height of both siblings at baseline was lower than the corresponding average age-related heights in the normal Japanese population, during ERT they were within, or close to, the average ± 1 standard deviation in the non-Fabry population. Their growth rate seemed to indicate catch-up growth. Other clinical symptoms were maintained at baseline levels.

Immunoglobulin G anti-agalsidase alfa antibodies were not detected in both sibling during ERT, and no infusion-associated reaction was observed. The treatment was generally well tolerated.

ERT was a safe and effective treatment for angiokeratoma and neuropathic pain for these two siblings with Fabry disease.

1. Introduction

Fabry disease is a congenital X-linked metabolic disorder caused by more than 650 genetic mutations of α-galactosidase A, a lysosomal enzyme [1].

The accumulation of the enzyme substrate glycosphingolipids (GSL) results in progressive, multi-organ impairment. The signs and symptoms emerge solely in childhood and adolescence, beginning with episodes of neuropathic pain and the appearance of angiokeratomas [2–4]. The incidence of cardiac involvement, renal dysfunction, and cerebrovascular problems increases with age and is the source of major morbidity and mortality [5,6].

Enzyme replacement therapy (ERT) for Fabry disease was introduced in Japan in 2004. Two forms of ERT are available for the treatment of Fabry disease: agalsidase alfa, which is manufactured in a human cell-line by gene activation [7,8] and agalsidase beta, which is manufactured in Chinese hamster ovary cells [9].

In clinical studies involving adult patients, ERT is associated with clinical benefits, including improvement in neuropathic pain [10,11], stabilization of kidney function [12,13], and improvement of cardiac structure and/or function [11,14,15].

Although clinical trial experience with ERT for the treatment of pediatric patients is limited, recent studies evaluating long-term treatment (up to 4 years of follow-up) with ERT in children with Fabry disease demonstrate that agalsidase alfa is well tolerated, with an efficacy profile consistent with that reported in adults [16–18].

In this report, we evaluated the safety and efficacy of ERT in two Japanese siblings with Fabry disease, who were diagnosed based on the presence of angiokeratomas alone, without any complaint of pain. Both siblings were followed up for 5 years. We were particularly interested in the effect of ERT on angiokeratomas and neuropathic pain.

2. Materials and methods

The pair of siblings was born to Japanese parents. Both boys have deletions of two bases (10208 and 10209) (Del 2b (#717-718),

Abbreviations: BPI, Brief Pain Inventory; Cre, creatinine; ERT, enzyme replacement therapy; EF, ejection fraction; eGFR, estimated glomerular filtration rate; GSL, glycosphingolipids; Gb3, globotriaosyl ceramide; LVMI, left ventricular mass index; MRI, magnetic resonance imaging.

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10208delAA) in exon 5 of the α-galactosidase A gene, resulting in a frame-shift followed by a downstream stop codon. Davies, J et al. reported that a patient with this mutation had the classical severe form of Fabry disease, characterized by pain in the extremities angiokeratoma, renal failure and cerebral and cardiovascular manifestations [19]. Their family tree is shown in Fig. 1.

2.1. Sib 1 (older brother)

The older boy was diagnosed with Fabry disease at the age of 13 years in March 2007 (when his height was 139.1 cm (−2.3 SD) and weight was 27.6 kg (−2.0 SD)). The diagnosis was initially suspected due to the presence of angiokeratomas. The level of α-galactosidase A activity in leukocytes was 0.2 nmol/mg protein/h, which is very low compared with the normal range of 49.8 to 116.4 nmol/mg protein/h. The levels of plasma and urine sediment Gb3 were 9.3 nmol/mL and 1634.7 nmol/g Cre, respectively.

The first symptom seemed to occur at the age of 1 year when it was diagnosed as juvenile rheumatoid arthritis. At the age of approximately 5 years, crust-like post-abrasion scarring and small dusky-red papules were noted on the lateral side of the knee. He tended to suffer from hypohidrosis when feeling hot, and in summer he was often asthenic and had a raised body temperature. At the age of 13 years, he was referred to the department of dermatology because of the small papules spread from his lower extremities to his chest.

2.2. Sib 2 (younger brother)

This boy is the younger brother of Sib 1. He was diagnosed with Fabry disease at the age of 11 years (when his height was 129.6 cm (−2.1 SD) and weight was 27.5 kg (−1.2 SD)), soon after the definitive diagnosis of his brother (Sib 1). The level of α-galactosidase A activity in leukocytes was 0.2 nmol/mg protein/h. The levels of plasma and urine sediment Gb3 were 13.9 nmol/mL and 1742.6 nmol/g Cre, respectively.

He was anhidrotic during his childhood, even during hot summer. He was not referred to a doctor because his parents thought both he and his brother (Sib 1) had a similar constitutional lack of sweating. When seen, he had cornea verticillata, but no angiokeratomas.

2.3. ERT treatment and clinical assessment

The two siblings received treatment with recombinant human agalsidase alfa (Replagal®, Dainippon-Sumitomo Pharma. Co.) diluted with physiological saline solution (JP), at a dose of 0.2 mg/kg every other weekly infused over a 40 min period.

Levels of plasma Gb3 and urinary Gb3 normalized for creatinine, and the level of immunoglobulin G anti-agalsidase alfa antibodies were determined by a central laboratory (SRL Medisearch, Tokyo, Japan) on a regular basis.

Cardiac structure and function were evaluated using chest X-ray, electrocardiography (ECG) and echocardiography (ECHO, two-dimensional and M-mode). Renal function was evaluated by serial measurements of estimated glomerular filtration rate (eGFR) using the new abbreviated Schwarz formula [20,21] and serial measurements of 24-hour albumin excretion. Fabry disease-related pain was evaluated using the Brief Pain Index (BPI) (average and worst scores). Ocular manifestations, auditory abnormalities and cerebrovascular manifestations were evaluated using ophthalmic slit-lamp microscopy, auditory brain-stem response (ABR) and cranial MRI, respectively. Safety/tolerability was also evaluated periodically.

3. Results

Both siblings were started on ERT in August 2007, receiving an infusion of agalsidase alfa 0.2 mg/kg every other week. They had been treated for 5 years by the end of August 2012.

The treatment was generally well tolerated. No infusion-associated reaction was observed, and immunoglobulin G anti-agalsidase alfa antibodies were not detected in either sibling.

3.1. Plasma and urine sediment Gb3

Plasma and urine sediment Gb3 levels were elevated at baseline in both siblings. Plasma Gb3 levels declined from 9.3 nmol/mL at baseline to 5.7 nmol/mL at 6 months in Sib 1, and from 13.9 nmol/mL to 5.8 nmol/mL in Sib 2 (Fig. 2a). Urine sediment Gb3 levels declined from 1634.7 nmol/g Cre at baseline to 607.3 nmol/g Cre at 6 months in Sib 1, and from 1742.6 nmol/g Cre to 376.7 nmol/g Cre in Sib 2 (Fig. 2b). Low levels of both parameters were maintained during ERT.

3.2. Skin symptoms

Sib 1 had angiokeratomas on the lateral side of his knee at baseline. After 48 months of therapy, the angiokeratomas had partially disappeared, with diminution of both size and number (Fig. 3).

After 3 months of therapy, the boys' hypohidrosis had improved. At baseline, Sib 1 experienced an increase in body temperature to

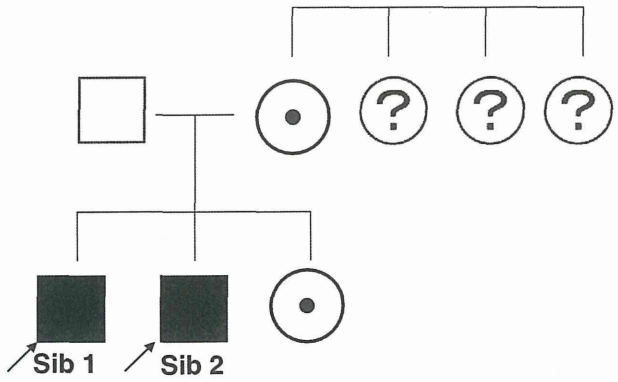

Fig. 1. Family pedigree of two Japanese siblings (Sib 1 and Sib 2) with Fabry disease.

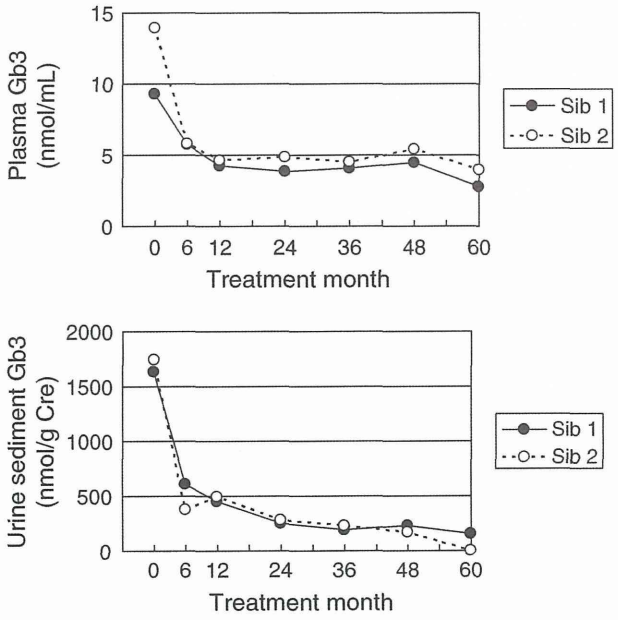

Fig. 2. Effect of agalsidase α treatment on plasma and urine sediment Gb3 levels for both siblings (Sib1 (●) and Sib 2 (○)).

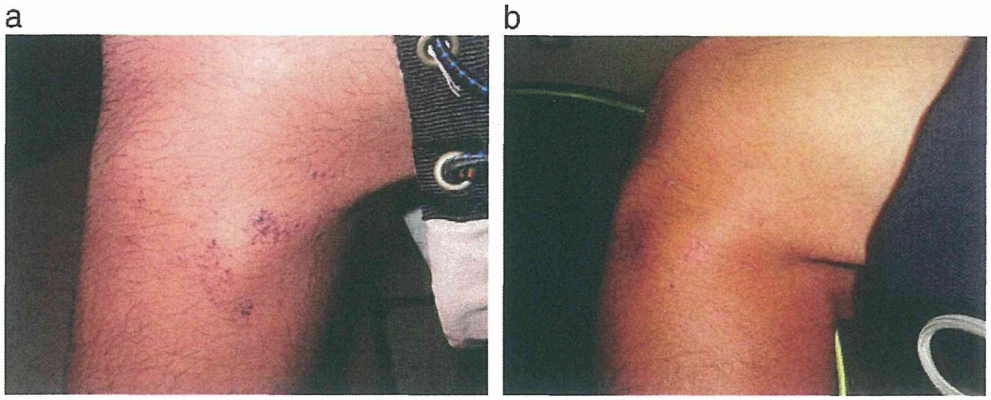

Fig. 3. Effect of agalsidase α treatment on angiokeratomas on the lateral knee. (a) Diffuse angiokeratoma at baseline, (b) diminution of both size and number of angiokeratomas after 48 months of treatment.

39–40 °C when he exercised. It was not so severe after 24 months of treatment, when it increased to a maximum of 37–38 °C when he exercised).

3.3. Pain

Neither of the siblings reported pain at baseline, and BPI scores for "pain at its worst" and "average pain" were both 0. Sib 1 and Sib 2 started to feel pain after 12 and 36 months of therapy, respectively (Fig. 4). However, the scores for "pain at its worst" decreased at 48 to 60 months for both siblings.

3.4. Growth rate

At baseline, the height of both siblings was lower than the corresponding average age-related height in normal Japanese males. During the period from 36 to 48 months of ERT, the height of Sib 1 was within the average ±1 standard deviation of the non-Fabry population, while that of Sib 2 was close to the average ±1 standard deviation at up to 60 months of ERT. Their growth rate seemed to indicate catch-up growth (Fig. 5).

3.5. Other symptoms

Values for eGFR, which were 130.6 and 132.2 mL/min per 1.73 m^2 at baseline, declined to 102.3 and 110.1 mL/min per 1.73 m^2 during ERT treatment after 60 months of therapy for Sib 1 and Sib 2, respectively (Fig. 6). Neither of the boys had proteinuria during the 5-year period.

Prior to the start of ERT, neither sibling showed any abnormalities in terms of auditory response, or cardiac and cerebrovascular symptoms. This remained the case after 60 months of treatment. In Sib 2, a radial deposit in the cornea was detected at baseline by ophthalmic slit-lamp microscopy. After 12 months of therapy, the cornea verticillata had completely disappeared (data not shown).

Table 1 summarizes comparative data for both siblings before and after 60 months of ERT.

4. Discussion

In this case study, we followed two Japanese siblings with Fabry disease, who were diagnosed after angiokeratomas were identified in the older sibling in the absence of pain. We evaluated the safety and efficacy of ERT when started in the relatively early stages of the disease.

The ability to sweat is often impaired in patients with Fabry disease [2,16], and this was evident in our cases. In both siblings, sweating was observed soon after the initiation of ERT, which motivated them to adhere to ERT. The resolution of a skin finding in one brother and disappearance of the corneal lesion in another indicates a positive response to ERT. Pain sensations were regained in both boys after 12 to 36 months of ERT, followed by a reduction after 48 to 60 treatment months.

The treatment was generally well tolerated. No infusion-associated reactions were observed, and immunoglobulin G anti-agalsidase alfa antibodies were not detected in either sibling. ERT was a safe and effective treatment for these two siblings with Fabry disease.

Angiokeratoma is considered the cutaneous hallmark of Fabry disease, and is present in 66% of male and 36% of female patients with Fabry disease [22]. In classically affected males, the earliest lesions

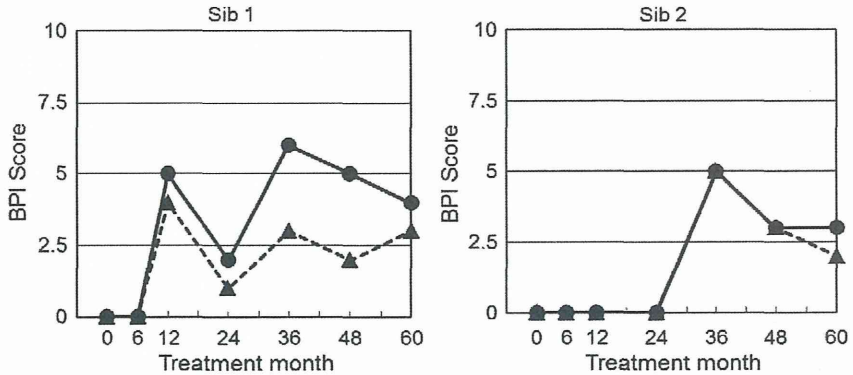

Fig. 4. The effect of agalsidase α treatment on Brief Pain Index scores, "pain at its worst" (●) and "average pain" (▲) in both siblings.

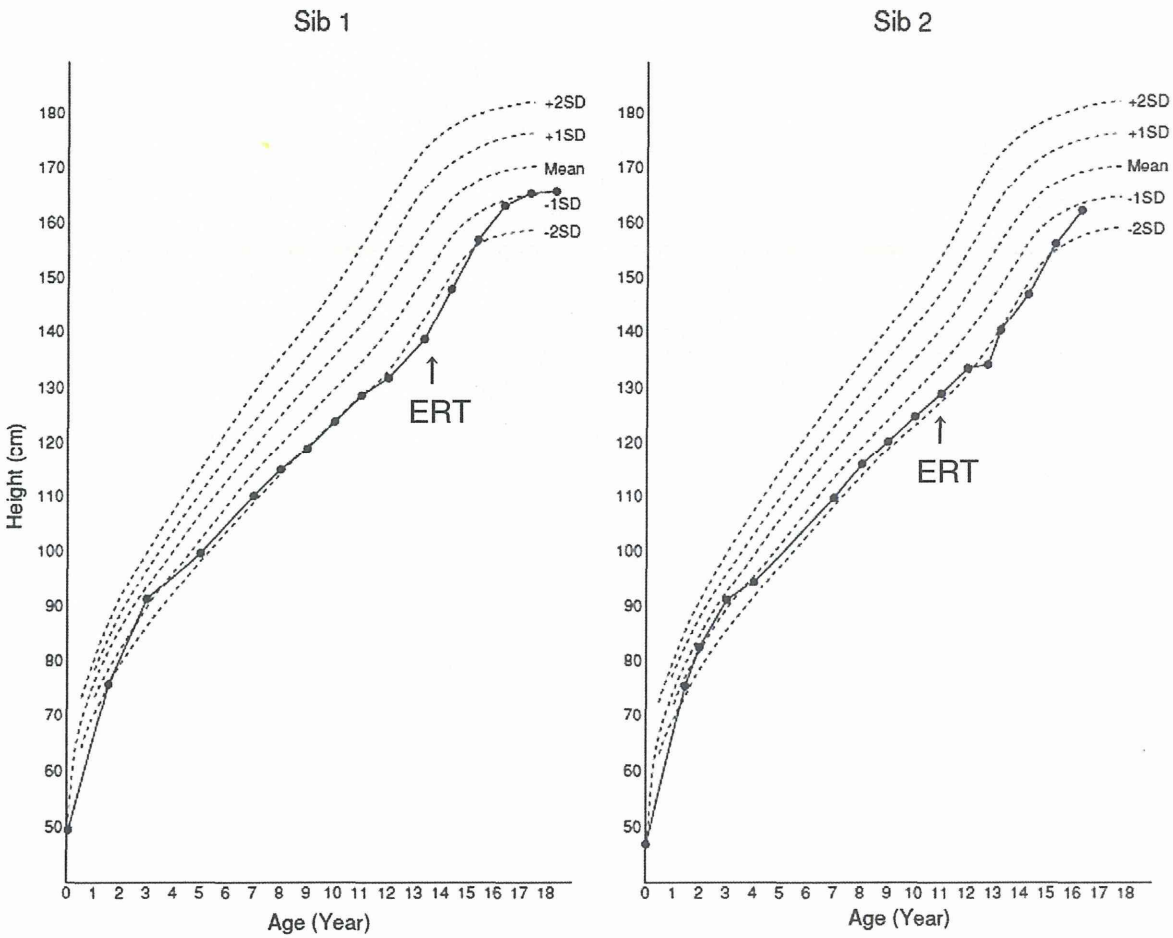

Fig. 5. Change in height for both siblings during agalsidase alfa treatment.

are observed during childhood on the hands, knees, elbows and flanks. Their number increases during adolescence, with lesions developing on the genitals, lumbosacral area, gluteal cleft and trunk [23]. However, the diagnosis of Fabry disease is often delayed until after end-organ damage has occurred [5].

In the case of the older sibling (Sib 1), it took 8 years until a definite diagnosis of Fabry disease was made when angiokeratomas were first noticed. This may be explained by the fact that both patients thought that the lack of sweating was constitutional. Furthermore, they were not referred to hospital because neither of the siblings complained of pain.

Serum α-galactosidase A activity can now be determined very easily. For a diagnosis of Fabry disease in males, it is sufficient to demonstrate low serum α-galactosidase A activity. On the other hand, in females genetic studies are mandatory because their α-galactosidase A activity may be normal [24]. This case highlights the need for vigilance within dermatology clinics; Fabry disease should be considered even if a solitary angiokeratoma is the only presenting feature.

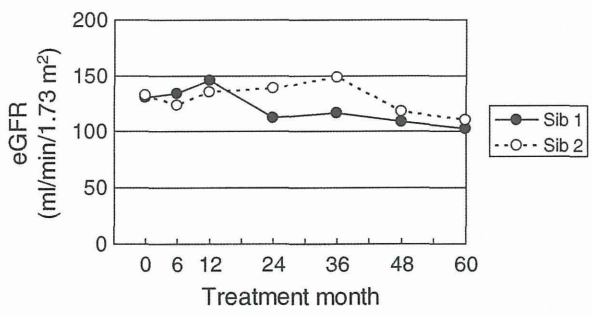

Fig. 6. Effect of agalsidase α treatment on eGFR levels for both siblings (Sib1 (●) and Sib 2 (○)).

The effect of ERT on the vasculopathy and retinal lesions in Fabry disease appears to be minimal [25]. However, recently, Fauchais et al. reported that ERT was effective at improving angiokeratomas [26]. We also observed an improvement in our patient. These dermatological lesions should be monitored regularly in order to clinically evaluate the efficacy of ERT.

Neuropathic pain was reported by ~60 and 80% of affected boys enrolled in the two Fabry disease registries, usually beginning in childhood, and by ~40 to 60% of affected girls, often a few years later than in boys [27,28]. Over time, neuropathic pain appears to diminish, perhaps due to progressive loss of nerve fibers, but can also increase [29,30].

In both of our siblings, pain sensation might be lost in time. Since Sib 1 has a history of juvenile rheumatoid arthritis, he might have complained of pain at a younger age. Neuropathic pain is often misdiagnosed as growth pain, malingering or neurosis, or is juvenile rheumatoid arthritis [31,32].

The primary neuropathic insult in Fabry disease is presumed to be due to a combination of factors that are linked with the accumulation of GSLs [33,34]. Accumulated GSLs can interfere with the function of critical proteins, such as ion channels, thereby causing nerve injury and dysfunction [35].

A BPI "pain at its worst" score above 5 is considered to indicate pain that interferes with activities of daily living (e.g., physical activity, mood, sleep, and social activities) [36]. Although BPI scores for "pain at its worst" of above 5 occurred in both siblings after the initiation of ERT,

Table 1
Comparison of clinical signs and symptoms between Sib 1 and Sib 2 at baseline and at 60 months after agalsidase α treatment.

	Sib 1 (diagnosed at 13 years old)		Sib 2 (diagnosed at 11 years old)	
	Baseline	60 months after ERT	Baseline	60 months after ERT
Plasma Gb3 (nmol/mL)	9.3	2.7	13.9	3.9
Urine sediment Gb3 (nmol/g Cre)	1634.7	156	1742.6	ND
Immunoglobulin G anti-agalsidase alfa antibody	Negative	Negative	Negative	Negative
Skin symptoms	+ (lateral knee)	Partially disappeared (at 48 months)	−	−
Angiokeratoma	Hypohidrosis	Improved	Hypohidrosis	Improved
Sweating abnormality				
Pain	No pain sensation	Started to feel pain (at 12 months) and decreased (at 48 months)	No pain sensation	Started to feel pain (at 36 months) and decreased (at 60 months)
Ocular symptoms	Normal	Normal	Cornea verticillata	Disappeared (at 12 months)
Auditory brain-stem response	Normal	Normal	Normal	Normal
Cardiac symptoms	26.3	29.1	26.7	24
LVMI ($g/m^{2.7}$)	6	8	5	6
Maximal wall thickness (mm)	64.3	65.2	68.4	78.3
EF (%)				
Renal findings	130.6	102.3	132.2	110.1
eGFR (mL/min/1.73 m^2)	0.04	0.02	0.04	0.04
Urinary protein (g/dL)				
Celebrovascular symptoms	Normal	Normal	Normal	Normal
Cranial MRI				

EF = ejection fraction; LVMI = left ventricular mass index.

they later decreased to below 4. Pain outcomes are reported to be improved by ERT in many clinical studies [10,37,38]. This reduction could be interpreted as improved small fiber function or alternatively as long term loss of sensory nerve function after an initial response to therapy.

Although renal findings of Fabry disease usually occur in adulthood, children with Fabry disease may present with mild renal dysfunction including hyperfiltration (>135 mL/min per 1.73 m^2), a small reduction in GFR, and microalbuminuria [18,39]. Hyperfiltration and microalbuminuria may be the initial signs of kidney dysfunction in Fabry disease. Early initiation of agalsidase alfa may have stabilized kidney function, although a longer-term study would be necessary to confirm this.

With regard to the renal [12,40,41] and cardiac [15] manifestations of Fabry disease, early initiation of treatment is expected to provide a better outcome. Long-term follow-up studies are required to confirm that initiation of ERT for Fabry disease during childhood can prevent the irreversible, life-threatening, organ damage that can occur during adulthood.

Finally, the mother and sister of our case study siblings are also at risk of developing the disease since they are female heterozygotes, and thus they should be followed up carefully.

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Enzyme augmentation therapy enhances the therapeutic efficacy of bone marrow transplantation in mucopolysaccharidosis type II mice

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ABSTRACT

Before the availability of an enzyme replacement therapy (ERT) for mucopolysaccharidosis type II (MPS II), patients were treated by bone marrow transplantation (BMT). However, the effectiveness of BMT for MPS II was equivocal, particularly at addressing the CNS manifestations. To study this further, we subjected a murine model of MPS II to BMT and evaluated the effect at correcting the biochemical and pathological aberrations in the viscera and CNS. Our results indicated that BMT reduced the accumulation of glycosaminoglycans (GAGs) in a variety of visceral organs, but not in the CNS. With the availability of an approved ERT for MPS II, we investigated and compared the relative merits of the two strategies either as a mono or combination therapy. We showed that the combination of BMT and ERT was additive at reducing tissue levels of GAGs in the heart, kidney and lung. Moreover, ERT conferred greater efficacy if the immunological response against the infused recombinant enzyme was low. Finally, we showed that pathologic GAGs might potentially represent a sensitive biomarker to monitor the therapeutic efficacy of therapies for MPS II.

1. Introduction

Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is an X-linked lysosomal storage disorder (LSD) caused by a deficiency in the activity of the lysosomal enzyme, iduronate-2-sulfate (IDS, EC 3.1.6.13), which degrades the glycosaminoglycans (GAGs), heparan sulfate and dermatan sulfate [1]. The widespread and progressive lysosomal accumulation of undegraded GAGs leads to a broad spectrum of clinical manifestations. These include skeletal deformities, cardiac valvular disease, cardiac hypertrophy, hepatosplenomegaly, coarse facial appearance, upper airway narrowing, hearing defect, enlarged tongue, retinopathy and CNS involvement [2]. These clinical symptoms significantly compromise the patients' quality of life.

Presently, two therapies are available to treat MPS II; one is enzyme replacement therapy (ERT) and the other is bone marrow transplantation (BMT). ERT has been shown to be effective at correcting aspects of the visceral disease [3–5] but not the CNS lesion as the enzyme dose not cross the blood–brain barrier [6,7]. ERT also has a limited impact on the bone and valvular lesions [8]. In addition, the need for repeated infusions of the enzyme is costly and confers a heavy burden on the MPS II patients [9].

BMT has been shown to be effective at treating several neuropathic LSDs such as MPS I, MPS VI, globoid-cell leukodystrophy, metachromatic leukodystrophy, Gaucher disease and others [10,11]. In contrast to ERT, BMT might address the CNS lesions associated with several LSDs by virtue of the migration of enzyme competent donor cells into the brain. Consequent secretion of the enzyme from these cells may allow for cross-correction of patients' enzyme deficient neuronal cells. Early studies suggested that BMT should not be indicated for MPS II patients due to disappointing outcomes, particularly the limited impact on CNS involvement [12–15]. However, recent report demonstrated the long-term efficacy of BMT in CNS involvement of an MPS II patient [16]. Thus, it is not evident if BMT should be indicated for MPS II. Moreover, there have been no animal studies showing the impact of BMT at reducing the level of GAGs in the brain.

Prior to the availability of ERT, some patients were treated by BMT with the hope of improving the visceral disease and CNS disease. Now

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that an approved enzyme is available for these patients, we asked if ERT could enhance the therapeutic effect of BMT. This has not been demonstrated for either human or murine MPS II. In this study, we examined the effectiveness of BMT at addressing CNS disease, as well as the relative merits of the combination of BMT and ERT in a mouse model of MPS II.

2. Methods

2.1. Animal husbandry

Female mice heterozygous for the X-linked allele ($IDS^{+/-}$) on a congenic C57BL/6 background were kindly provided from Joseph Muenzer (University of North Carolina, Chapel Hill) [17]. The carrier females were bred with male wild type (WT) mice of the same genetic background strain, producing hemizygous IDS knock-out males (MPS II mouse model, $IdS^{-/0}$). The genotypes of all offspring were determined by polymerase chain reaction analysis of DNA obtained from a tail snip. B6.SJL-ptprca mice congenic at the CD45 locus ($CD45.1^{+}CD45.2^{-}$) were purchased from Sankyo Labo Service (Tokyo, Japan), and bred with female C57BL/6 ($CD45.1^{-}CD45.2^{+}$) WT, producing C57BL/6 ($CD45.1^{+}CD45.2^{+}$) donor mice. These mice were used as donors. Animal husbandry and all procedures in the animal experiments were approved by The Animal Care Committee at The Jikei University School of Medicine.

2.2. BMT

BMT was performed as previously described with minor modification [18]. Bone marrow cells were harvested from femurs and tibias of male C57BL/6 ($CD45.1^{+}CD45.2^{+}$) mice (8–12 weeks of age). The bone marrow cells (2.0×10^6) were transplanted to lethally irradiated (9Gy) recipient MPS II mice intravenously. Irradiation was carried out using the Hitach-MBR1520R irradiator (Hitachi, Tokyo, Japan). To confirm engraftment, the peripheral blood was collected from treated mice (2 times: 12 weeks and 27 weeks after transplantation). Flow cytometric analysis was carried out using MACSQuant® Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using MACSQuantify® Software (Miltenyi Biotec). Briefly, peripheral blood cells were stained with fluorescein isothiocyanate-conjugated anti-murine CD45.1 and allophycocyanin-conjugated anti-murine CD45.2 (eBioscience, San Diego, CA, USA). The each lineage was distinguished by using the corresponding phycoerythrin-conjugated antibody: (Bcell-CD45R, Tcell-CD3e, Granulocyte-Ly6G, Macrophage-CD11b, eBioscience). The donor-derived cell engraftment was determined as the percentage of $CD45.1^{+}CD45.2^{+}$ cells.

2.3. ERT

The MPS II mice were administered 0.5 mg/kg human IDS (Idursulfase, Shire HGT Pharmaceuticals, Cambridge, MA, USA, generously gifted from Genzyme Japan Co., Ltd. in Tokyo) via a tail vein once a week.

2.4. Therapeutic regimen

Therapeutic regimen was shown in Fig. 1. There are 3 treatment groups, BMT group, ERT group and BMT + ERT group. Control groups consisted with untreated MPS II mice and WT mice. In ERT group, ERT started at 9 weeks of age with total 27 times. In BMT group, BMT was carried out at 9 weeks of age. In BMT + ERT group, BMT was performed at 9 weeks of age and ERT was initiated at 12 weeks after BMT with total 15 times. All mice were measured the body weight and collected the blood and urine samples at various time points. In ERT and BMT + ERT groups, blood was harvested just prior to ERT every time. Y-maze test was carried out in all treatment groups at between 26 and 27 weeks after treatment, and sacrificed for tissue biochemical and pathological assays at 27 weeks after initiation of treatment. The mice in ERT and BMT + ERT groups sacrificed 1 week after last ERT.

2.5. Serum and tissue IDS activity

IDS activity was determined in serum and homogenized tissue, as previously described using the artificial substrate 4-methylumbelliferyl-alpha-iduronide-2-sulfate (MU-αIdu-2S) (Moscerdam Substrates, Oegstgeest, Netherlands) [19]. Protein concentrations were determined using the BCA protein assay kit (Thermo Fisher Scientific, IL, USA). The tissue IDS enzyme activity was expressed as nmol/4 h/mg protein. The serum IDS activity was expressed as % activity of WT mice.

2.6. Total GAGs assay

Total GAG (tGAG) concentration in urine and tissue extracts was quantified by the Wieslad® sGAG quantitative Alcian blue-binding assay kit (Euro-Diagnostica, Malmö, Sweden) as previously described [19]. The urine creatinine was assayed using a commercially available kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The urinary and tissue GAG amount was expressed as μg GAG/mg creatinine and μg GAG/mg protein, respectively.

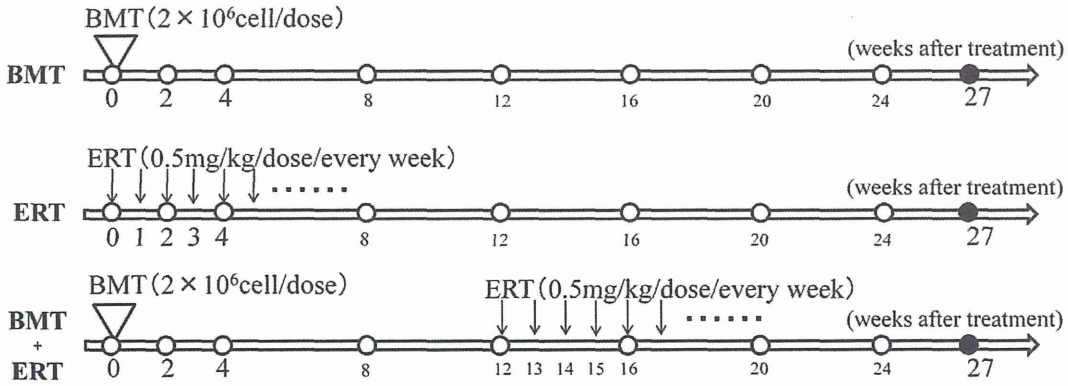

Fig. 1. Therapy experimental design. The picture shows diagram of therapeutic regimen. The therapeutic groups included BMT only, ERT only, and the combination of BMT and ERT in MPS II mice. Downward triangles mark the initiation of BMT. The solid arrows indicate IDS administration. Open circles mark time points of blood and urine collection for IDS activity, GAG detection, and anti-IDS antibody assays. The filled circles show the time point of sacrifice. In BMT groups, BMT was carried out at 9 weeks of age. In ERT group, ERT was initiated at 9 weeks of age followed by weekly infusion total 27 times. In ERT + BMT groups, BMT was carried out at 9 weeks of age and ERT was added 12 weeks after BMT. Thereafter, ERT was continued weekly for a total of 15 times. All mice were sacrificed 27 weeks after initiation of treatment.

2.7. Pathologic GAGs assay

The pathologic GAGs (pGAGs) assays were performed by using the Sensi-Pro Non-Reducing End (NRE) heparan sulfate assay as described [20]. The Sensi-Pro assay measures only lysosomal accumulated heparan sulfate caused by the deficient activity of IDS by specifically quantifying the 2-O sulfated non-reducing end derived after heparin lyase digestion. Briefly, tissue samples were homogenized and the GAGs were isolated by anion exchange chromatography and digested with heparin lyase (IBEX Technologies, Montreal, Canada). Enzymatically depolymerized MPS II specific NRE, 2-sulfoiduronic linked to N-sulfoglucosamine-6-sulfate (I2S6), tagged with [$^{12}C_6$] was measured according to standard curves of saturated heparan sulfate tagged with [$^{13}C_6$], derived commercially available standard unsaturated disaccharides. This measurement was performed by using Liquid Chromatography–Mass Spectrometry and the data were represented as average of triplicates.

2.8. Pathologically analysis

The mice were euthanized using pentobarbital and perfused with 4% paraformaldehyde and 2% glutaraldehyde (Wako Pure Chemical Industries, Ltd.) in 0.1 M PBS through the heart. The liver, heart, spleen and kidney were removed and immersed in the same fixative solution for a minimum of 2 h. Tissue samples were postfixed in 1% osmium tetroxide, and embedded in epoxy resin. The sections were cut at 1 μm thickness, stained with toluidine blue, and assessed to detect large distended lysosomes using light microscopy (BX50; Olympus Optical Co, Ltd., Tokyo, Japan).

2.9. Assay for rhIDS-specific IgG

IgG antibodies recognizing rhIDS were assayed using an enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well plates Nunc-Immuno palate MaxiSorp® (Nunc, Roskilde, Denmark) were coated with 10 μg of rhIDS in PBS overnight at 4 °C. The plates were blocked by adding 100 μl PBS/1% bovine serum albumin and incubating for 5 h at room temperature. After this step, the wells were washed with PBS/0.05% Tween 20. The serum samples from mice were diluted 100-fold with PBS/1% bovine serum albumin, and 100 μl diluted serum was added to each well and incubated for 1 h at room temperature. The plate was then washed with the same buffer and reacted with 100 μl of 5000-fold diluted peroxidase-conjugated anti-mouse IgG Ab (Kirkegaard & Perry Labs, Gaithersburg, MA, USA). After incubation for 30 min at room temperature, the plates were washed again and color was generated by the addition of 3,3′,5′,5′-tetramethylbenzidine substrate reagent (Kirkegaard & Perry Labs) for 10 min at room temperature. The reaction was stopped by adding 100 μl of 0.6 N H_2SO_4, and the optical density was measured at 450 nm using an ARVOMX/Light plate reader (PerkinElmer, Waltham, MA, USA). The Ab titer was calculated using mouse anti-human IDS monoclonal antibody as a standard (generously gifted by JCR pharmaceutical Co., Ltd.).

2.10. Statistical analysis

Data were assessed using GraphPad Prism software (GraphPad software, Inc., La Jolla, CA, USA). T-test or one-way ANOVA with Tukey–Kramer's post test was used. Significance was considered to be $P < 0.05$.

3. Results

3.1. Engraftment of donor cells in mice treated with BMT and a combination of BMT and ERT

Donor cell engraftment in mice subjected to BMT and BMT + ERT was analyzed at 12 weeks and 27 weeks following BMT (Table 1).

Table 1
Donor-derived cell engraftment in each lineage in BMT and BMT + ERT groups.

	B cell (%)[a]	T cell (%)[a]	Granulocyte (%)[a]	Macrophage (%)[a]
12 weeks after BMT				
BMT	95.72 ± 4.51	78.99 ± 5.39	87.39 ± 4.64	89.6 ± 7.88
BMT + ERT	96.41 ± 1.64	79.95 ± 4.19	89.8 ± 3.84	92.89 ± 3.8
27 weeks after BMT				
BMT	93.9 ± 8.41	78.34 ± 5.8	92.36 ± 6.58	91.28 ± 9.44
BMT + ERT	95.46 ± 3.04	85.48 ± 4.3	94.82 ± 3.05	89.1 ± 4.174

[a] Percentage of donor-derived cells in peripheral blood at 12 or 27 weeks after BMT. The individual values are shown as mean values ± SD (BMT: n = 5, BMT + ERT: n = 5).

Approximately 90% donor cell engraftment in each lineage cell was achieved at 12 and 27 weeks in animals that received BMT alone or in combination with ERT. Percent engraftment of T cell lineage was lower than for other cell lineages probably because of the longer half life time of pre-existing recipient T cells.

3.2. Serum IDS activity in mice treated with BMT, ERT or a combination of BMT and ERT

Serum IDS activities at various time points were assayed following treatment (Fig. 2). MPS II mice treated by BMT alone showed a rise in IDS activity beginning at 2 weeks post-transplantation which reached 25% of WT levels at 20 weeks ($P < 0.001$ vs. untreated MPS II mouse, NT). Animals treated with ERT alone had no detectable serum IDS activity as the infused IDS was rapidly cleared from circulation. Serum IDS levels in mice treated by the combination of BMT and ERT exhibited a profile that was similar to mice treated by BMT alone ($P < 0.001$ vs. NT, $P > 0.05$ vs. BMT). As may be expected the serum IDS enzymatic activity did not increase even after initiation of ERT as the infused enzyme was rapidly cleared from circulation. These observations indicate that the donor cells were stably engrafted in the BMT treated mice and that they excreted IDS for a sustained period.

3.3. Tissue IDS activity in mice treated by BMT or ERT alone and combination of BMT and ERT

Twenty-seven weeks after treatment by BMT or ERT or the combination, IDS activity in various tissues of MPS II mice was analyzed. In the ERT treated group (Fig. 3A), no significant increase in IDS activity

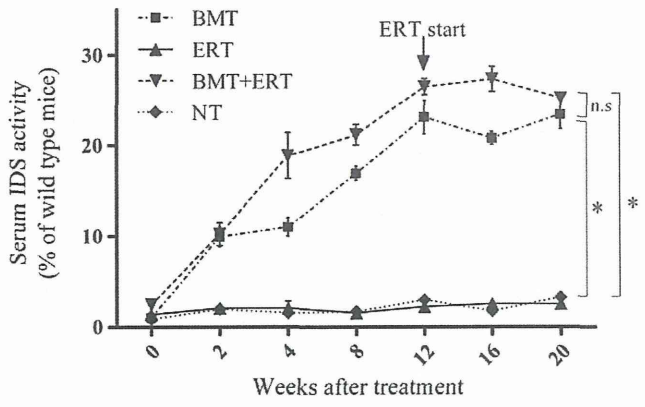

Fig. 2. Sustained IDS activities in serum from BMT received MPS II. IDS activity in serum from MPS II mice in three treatment groups, WT mice and NT mice was measured as described in Methods. The IDS activities were assayed duplicate. Arrows indicate start point of ERT in BMT + ERT group. IDS activity is expressed as % of age matched WT mice (data of WT mice is not shown in figure). The IDS activities at each time point are shown as mean ± SEM (each group: n = 5). The difference of enzyme activities at 20 weeks after treatment was compared by one-way ANOVA among 3 treatment groups and NT group. Asterisk indicates $P < 0.001$. "n.s" indicates no significant difference.